Regulation of EGF Receptor Signaling by a Cbl Dimerization Interface

A Senior Thesis

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by
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CERTIFICATE OF APPROVAL

SENIOR THESIS

This is to certify that the Senior thesis of

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has been approved by the Examining Committee for the thesis requirement for graduation with research distinction at the August 2011 graduation.

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Abstract

Epidermal growth factor receptor (EGFR) overexpression is associated with many tumors, including those of the breast, lung and brain. Understanding how endogenous suppressors of EGFR signaling work could reveal new therapeutic interventions for cancer patients. The ubiquitin ligase Cbl targets EGFR for destruction by ubiquitinating Sprouty2, EGFR, and Hrs at distinct endocytosis checkpoints. These events increase receptor delivery to lysosomes. The Lill laboratory has reported that Cbl's RING finger (RF) tail domain is essential for degradative targeting of EGFR. My project goal is to identify the mechanism through which RF tail amino acids (aa) act. Based on available crystal structure data, our lab hypothesized that the RF tail mediates the formation of Cbl homodimers with enhanced ubiquitin ligase activity, and that the dimer interface comprises the RF tail of one Cbl molecule and aa 286-354 of a second. In this model, Cbl homodimers are critical for negotiating the Sprouty2 and Hrs trafficking checkpoints of the endocytic pathway. For my project, I analyzed mammalian cells expressing Cbl mutants that were substituted in aa 286-354 (one side of the putative dimer interface). The experiments included assays of EGFR downregulation, in which intact cells were immunostained for fluorescent detection of surface EGFR molecules (red) through flow cytometry. By comparing green fluorescent protein (GFP)-tagged wild-type and mutant Cbl proteins, I determined their relative abilities to program EGFR for removal from the cell surface. The data revealed that specific amino acids within the putative 286-354 interface region critically control EGFR surface levels, as their mutation compromised EGFR downregulation. These results support the hypothesis that the RF tail and aa 286-354 regions of Cbl jointly regulate EGFR fate through their interaction at a Cbl dimer interface. Therefore, it may be possible to develop molecular

tools that modulate Cbl dimer formation by mimicking one or the other surface at the interface, thereby altering EGFR signaling in EGFR-positive tumors.

Introduction

Cellular hyperproliferation is one of the characteristics of many epithelial cancers such as those of the lung, breast, and brain. Frequently, this is related to an abnormal increase in the activity of the epidermal growth factor receptor (EGFR) (5, 6). Balanced regulation of receptor tyrosine kinase activity is necessary for normal growth and development: reduced EGFR signaling is fatal in knockout mice (9), while excessive signaling can lead to cancer (3). Research into how normal cells regulate EGFR may reveal new molecular targets for innovative anticancer therapies.

The biological processes of endocytic trafficking and lysosomal degradation must be regulated in order to maintain normal EGFR levels and signaling. EGFR's trafficking to lysosomes is one mechanism through which receptor signaling is suppressed (Figure 1). Along this path, ligand-activated EGFR dimerizes, becomes ubiquitinated at the cell surface, and is internalized on the limiting membranes of early endosomes. The vesicles fuse and mature as their luminal pH becomes more acidic. During this phase, EGFR is either recycled to the cell surface or sorted onto luminal membranes within the developing multivesicular bodies (MVBs). In this way, activated receptors are segregated from their cytosolic signaling partners. The sorting onto luminal membranes facilitates the destruction of the receptors within lysosomes, because the MVBs mature subsequently through fusion with lysosomes and incorporate destructive proteases and lipases. Lysosomal degradation is an unambiguous mechanism for EGFR signaling termination (1, 7, 12).

The Lill laboratory has been investigating the nature of molecular complexes that regulate EGFR endocytic trafficking and signaling downstream of internalization. Their research has

revealed important roles for the receptor-associated protein Cbl, which mediates the fusion and maturation of endosomes (15).

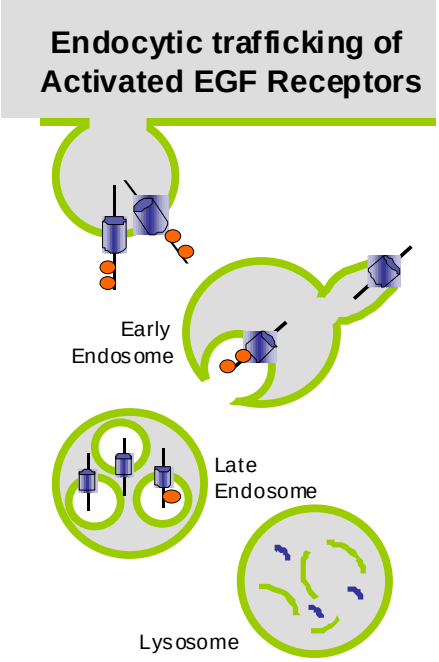

Figure 1. The endocytic pathway leading to EGFR degradation. Ligand-activated EGFR is: ubiquitinated at the cell surface; internalized on vesicles that fuse with early endosomes; either recycled to the cell surface or sorted onto luminal membranes that are internalized from endosome limiting membranes. Luminal vesicle formation, initiated at early endosomes, is complete at late endosomes (multivesicular morphology). Lysosomal delivery allows for destruction of internal membranes and their cargo.

This undergraduate thesis addresses the following question: What molecular or structural mechanisms are involved in Cbl's regulation of EGF receptor degradation? According to research from the Lill laboratory, a short stretch of amino acids in Cbl, known as the RING finger tail, is vital for EGF receptor regulation. Single amino acid substitutions of V431A or F434A in this domain result in Cbl proteins that cannot regulate the receptor appropriately (15). The V431A mutant blocks activated EGFR at the cell surface, so it never proceeds to endosomes. However, the F434A mutant allows the receptor/Cbl complexes to move to the limiting membrane of endosomes, but not to lysosomes. This phenomenon indicates that Cbl F434A is a unique mutant, which can be analyzed further at the molecular level to show how normal (wild-type) Cbl controls the endosomal fusion checkpoint and luminal vesicle formation.

Cbl has been suggested to control EGFR endocytosis at the level of receptor internalization (16) and to regulate receptor fate post-internalization (15). Cbl is an evolutionarily conserved, 906 amino acid suppressor of EGFR signaling (17-19). Its ligand-induced association with EGFR is direct: Cbl's variant SH2 domain binds EGFR phosphotyrosine 1045. Cbl's recruitment to EGFR results in receptor ubiquitination that is mediated by Cbl's E3/ubiquitin ligase activity. Others have suggested that the receptor ubiquitination is sufficient to program activated EGFR for degradation. However, our recent studies indicate that additional Cbl activities, functioning downstream of EGFR ubiquitination, also control receptor fate (15).

We reported that amino acid 434 is the C-terminal limit of the Cbl residues sufficient to enhance EGFR downregulation (13). Cbl 1-434 comprises a tyrosine kinase-binding (TKB) domain, linker region, RING finger (RF), and the majority of the RF tail (Figure 2). The RF tail spans residues 420-436 (Figure 3). The domain was structured in a co-crystal of Cbl residues 47-434 with the E2/ubiquitin-conjugating enzyme UbcH7 (14). The crystal structure suggested that RF tail residues V431 and F434 play no role in either intramolecular or intermolecular Cbl/UbcH7 interactions (Figures 2,4). However, both amino acids proved to be critically important functional regulators of Cbl-mediated EGFR trafficking and degradation (15). *Why?*

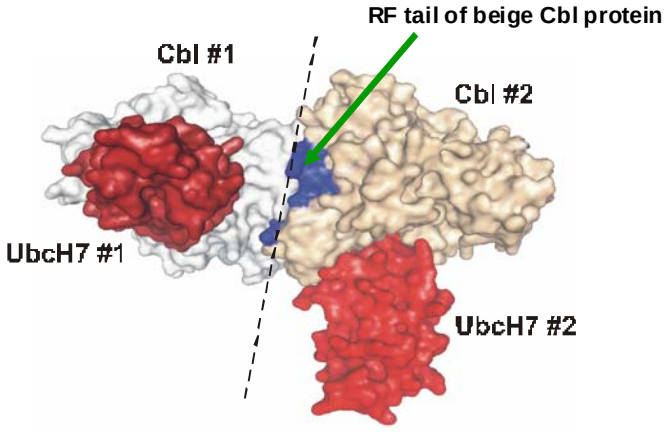

Figure 2. **RF tail residues form one side of an interface between Cbl/Cbl complexes within the Cbl/UbcH7 crystal structure.** The globular forms of proteins present within the crystal are shown. The white and beige molecules represent adjacent Cbl proteins. The blue segment corresponds to the RF tail of the beige Cbl protein. The red molecules are E2/UbcH7 proteins that carry ubiquitin and bind to the E3/Cbl.

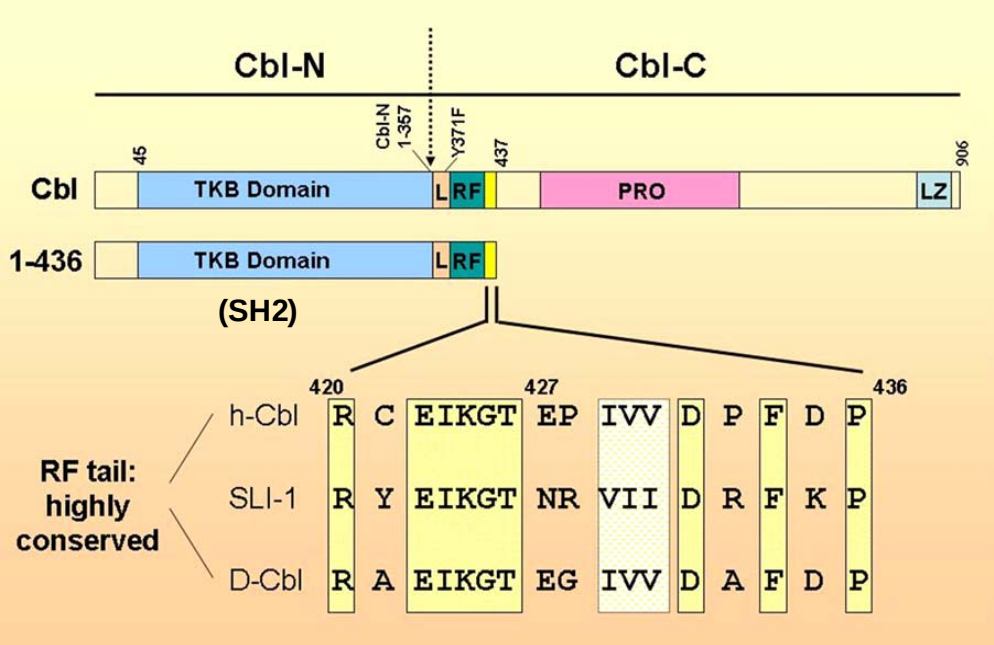

Figure 3. **Conserved Cbl domains.** Cbl-N contains the SH2 domain known as the tyrosine kinase-binding (TKB) domain. The linker (L), RING finger (RF), and RF tail (T) regions complete the first 436 amino acids of Cbl that are evolutionarily conserved in human, mouse, *C. elegans* and *Drosophila*. Expanded below the schematic representation of Cbl 1-436 are the RF tail sequences from human (h-Cbl), *C. elegans* (SLI-1), and *Drosophila* (D-Cbl). Identical residues are shown in yellow boxes, while the similar small hydrophobic aa are in the lighter box.

To address this question, we re-analyzed the reported structural data. A second look at the structure suggested that Cbl molecules might form dimers (Figure 2) whose interface comprises the RF tail of one Cbl and a different region of the second Cbl molecule (Figures 2,3). We therefore undertook structure-function studies to determine whether other putative Cbl interface residues are critical for EGFR regulation, as suggested by Figure 4. Single amino acid substitution mutants of full-length Cbl were tested for their ability to enhance EGFR downregulation from the cell surface, a key step in the degradative trafficking of the receptor which is easily assayed. Our results are presented here.

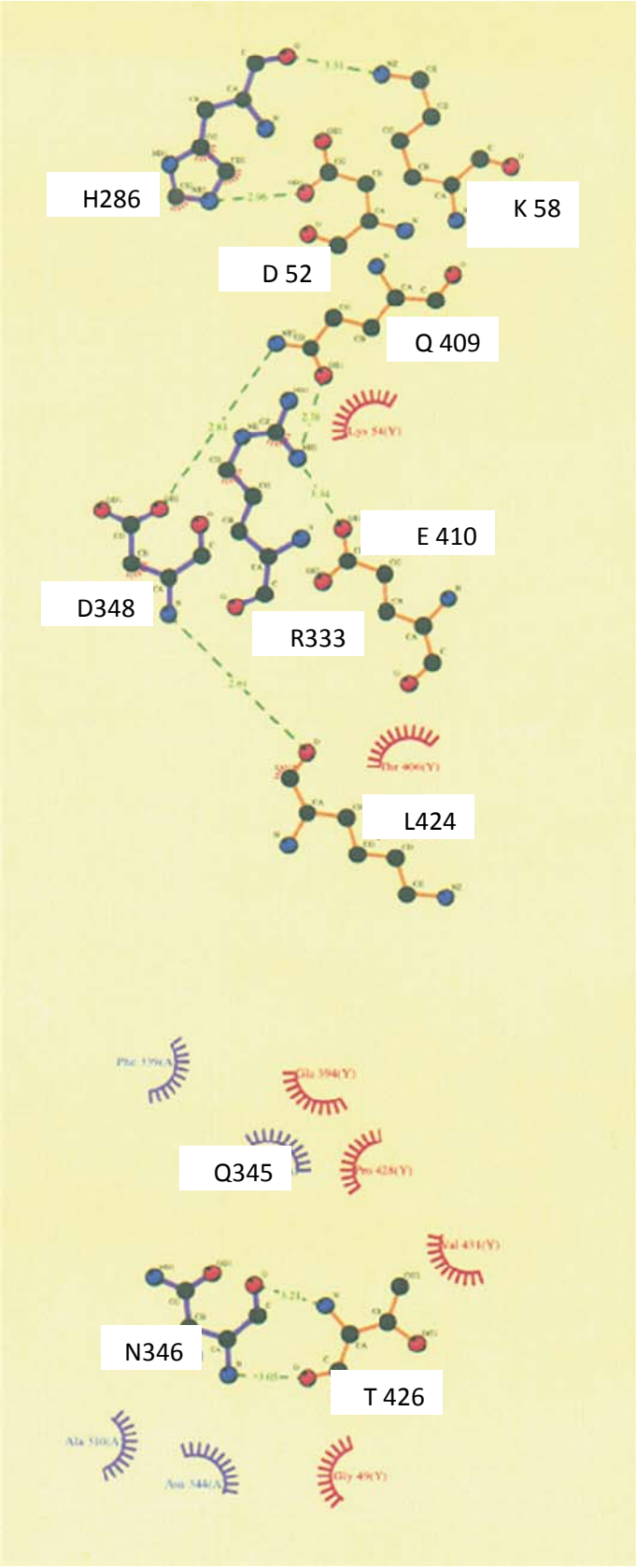

Figure 4. Interactions at the interface of adjacent Cbl proteins in the solved crystal structure. RF tail residues include L424, P428, and V431. Residues on the other side of the interface include H286, R333, N346, Q345 and D348, which apparently participate in intermolecular hydrogen bonding.

Objective:

The goal of my project was to determine why mutations within the RING finger tail have an impact on EGFR downregulation. A molecular mechanism for this regulation has been hypothesized by the Lill Laboratory.

Hypothesis:

Cbl controls EGF receptor downregulation by forming Cbl/Cbl homodimers that possess enhanced ubiquitin ligase activity. The dimer interface consists of the RING finger tail region of the first Cbl molecule and amino acids 286-354 of the adjacent Cbl molecule.

Rationale:

Pavletich and colleagues solved the complex of Cbl and an E2 (UbcH7) in 2000 (14). Intermolecular protein interfaces between Cbl and UbcH7 were proposed to be potentially important for Cbl's activity. However, the investigators didn't consider Cbl/Cbl interactions within the crystal. The Lill Laboratory, in collaboration with the crystallography lab of S. Ramaswamy at the University of Iowa, reexamined the Cbl-E2 crystal data and observed that an apparent interface exists between adjacent Cbl molecules (Figure 2). The putative interface involves the RING finger tail and residues 286-354 from distinct Cbl proteins.

The Lill laboratory had already found that RING finger tail residues V431A and F431A were critical in regulating EGF receptor trafficking and fate (15). Knowing that residues on one side of the Cbl dimer interface were important for Cbl function, we wished to test whether mutations in the other side of the Cbl interface would also compromise Cbl's ability to regulate endocytic

trafficking of EGFR. A panel of mutant Cbl expression constructs was developed, each specifying an alanine substitution at the Cbl interface opposite the RF tail. The full panel of mutations is summarized in Table 1, below.

Table 1. Mutations generated in full-length Cbl for this study, and the sense mutagenic primer sequences that were used to make them

	Mutation	Sequence of the corresponding sense mutagenic primer
1.	H286A	5'-CTCGGCTCCAGAAATTCATT**GCC**AAACCTGGCAGTTATATC-3'
2.	A310L	5'- GCTATTGGGTATGTTACT**CTT**GATGGGAACATTCTCC -3'
3.	D311A	5'- GGTATGTTACTGCT**GCT**GGGAACATTCTCC -3'
4.	F332A	5'- CACTGATTGATGGC**GCC**AGGGAAGGCTTC -3'
5.	R333A	5'-CACTGATTGATGGCTTC**GCG**GAAGGCTTCTATTTG-3'
6.	F339A	5'- GGAAGGCTTCTATTTG**GCT**CCTGATGGACGAAATC -3'
7.	N344A	5'- GTTTCCTGATGGACGA**GCT**CAGAATCCTGATCTG -3'
8.	Q345A	5'-GTTTCCTGATGGACGAAAT**GCG**AATCCTGATCTGACTGG-3'
9.	N346A	5'-GATGGACGAAATCAG**GCT**CCTGATCTGACTG-3'
10.	P347A	5'- GGACGAAATCAGAAT**GCT**GATCTGACTGGC -3'
11.	D348A	5'-GAAATCAGAATCCT**GCT**CTGACTGGCTTAT-3'
12.	T350A	5'- CAGAATCCTGATCTG**GCT**GGCTTATGTGAAC -3'
13.	E354A	5'- GACTGGCTTATGT**GCA**CCAACTCCCCAAG -3'

For the undergraduate research project, a subset of the mutant expression constructs was selected for functional characterization. These included Cbl D348A, H286A, N346A, and R333A, which altered amino acids that appeared to participate in intermolecular hydrogen bond interactions. Q345A was added to the analysis to represent a nearby residue engaged only in a hydrophobic interaction.

If any of these residues were vital for normal EGFR regulation, we would expect that their substitution by alanine would compromise Cbl's function. For this study, the Cbl function assayed was the ability of Cbl to enhance EGFR downregulation from the cell surface (8,13,15).

Techniques/Description of Approach:

The EGFR downregulation assay is a live-cell immunostaining approach that detects only the receptors on the cell surface. Briefly, a parental cell line is transiently transfected to express the control and experimental proteins of interest. Replicate cultures are incubated for several days to allow for expression of the proteins. On the day of the experiment, the cultures are serum-starved to make all EGFRs inactive, which results in their accumulation on the cell surface. One of the replicate plates from each set is harvested without addition of EGF; this is the "0 minute" timepoint at which surface EGFR levels are maximal, so it serves as the reference control for its stimulated counterparts. Additional plates from each set are EGF-stimulated for different times, so that the ligand-induced reduction in surface EGFR levels can be graphed over an experimental time course of 40 minutes.

When the positive-control wt Cbl protein is expressed by the cells, the downregulation curve is shifted downwards relative to the GFP "null" control curve which reflects the activity of only

the endogenous Cbl protein. The curves of the experimental mutant proteins are compared to the control curves to determine whether they downregulate EGFR like wt Cbl, or if they are compromised for function and more closely resemble the GFP control curve. If a mutant fails to downregulate EGF receptor like wild-type Cbl, its experimental curve would be statistically different from that of the Cbl-expressing cells. The mutants would be scored as abnormal and then studied in further trafficking regulation experiments.

Methods:

Site-directed mutagenesis

The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to introduce mutations into the plasmid pcDNA3-GFP Cbl wt (Figure 5), resulting in the substitution of various residues by alanine. The following reagents were used for the preparation of mutated constructs: 5 microliters of 10X PCR buffer, 1 microliter of 10mM dNTPs, 1 microliter of forward and reverse primer each at concentration of 125 nanograms/microliter, 18 nanograms (1 microliter) of pcDNA3-GFP Cbl wt, 1 microliter of Quickchange PfuTurbo DNA polymerase (2.5 U/microliter), and 38.5 microliters of deionized distilled water, bringing the final volume to 50 microliters. PCR settings were as follows: segment 1 – (1 cycle), temperature: 95° Celsius for 2 minutes; segment 2 – (18 cycles), temperature: 95° Celsius for 20 seconds, 60° Celsius for 10 seconds, 68° Celsius for 5 minutes ; segment 3 – (1 cycle), temperature 68° Celsius for 5 minutes, then hold at room temperature. After cycling, the reaction tubes were placed on ice for 2 minutes to bring the reaction temperature down, and then 1 microliter of *Dpn* I restriction enzyme (10U/microliter) was directly added to the amplification reaction to cleave methylated restriction sites. The enzyme activity degraded the parental strand/template plasmid without affecting the amplified PCR products that bore the mutations. Positive and negative controls were included. The positive control was DNA from the QuickChange kit that confers ampicillin resistance; the negative control consisted of all reagents without any added DNA. After *Dpn* I addition, the reaction mixes were swirled and incubated at room temperature for one hour.

XL1-Blue supercompetent cells were thawed on ice and cell aliquots were used for bacterial transformation by the DNAs. For each transformation reaction, 1 microliter of *Dpn*I-treated

digest reaction was added to 50 microliters of supercompetent cells in a 5 mL BD Falcon polypropylene tube. The transformation reaction was incubated on ice for 30 minutes and then heat pulsed for 45 seconds at 42° Celsius, followed by incubation on ice for 2 minutes. Afterwards, 0.5 milliliters of pre-heated LB broth was added to the reaction mixture and the cells were incubated at 37° Celsius for expansion of the bacterial cells. Aliquots of 100 microliters of culture were plated onto LB/ampicillin plates, which were incubated at 37° Celsius for 18-20 hours. Colonies were picked for expansion, DNA preparation, and DNA sequence analysis. All resulting constructs were sequenced using a primer, 5'-CCACATGCTGGCAGAACT – 3', corresponding to the sense sequence of Cbl which lies 5' to the mutated region of interest. Sequencing was performed at the Ohio State Nucleic Acid Shared Resource Facility (Columbus).

To generate a plasmid encoding the R333A mutation, PCR-based mutagenesis was used because site-directed mutagenesis had failed to produce any colonies on ampicillin/LB plates, despite troubleshooting experiments that modified reaction temperature settings and DMSO content. For PCR-based mutagenesis, four primers were used (2 per reaction; with reaction 1 generating a PCR fragment spanning sequences 5' to and through the mutation and reaction 2 generating a PCR fragment spanning sequences 3' to and through the mutation). The two reaction products were GeneCleaned, mixed, and then subjected to a new cycle of PCR to generate a single product incorporating sequences from both initial PCR fragments. The final product was digested with *Eco*RI and *Sac*II and then cloned into digested pcDNA3-GFP Cbl wt vector with compatible ends. To check the resulting ligation products, derived plasmids were sequenced for the presence of alanine-encoding mutations.

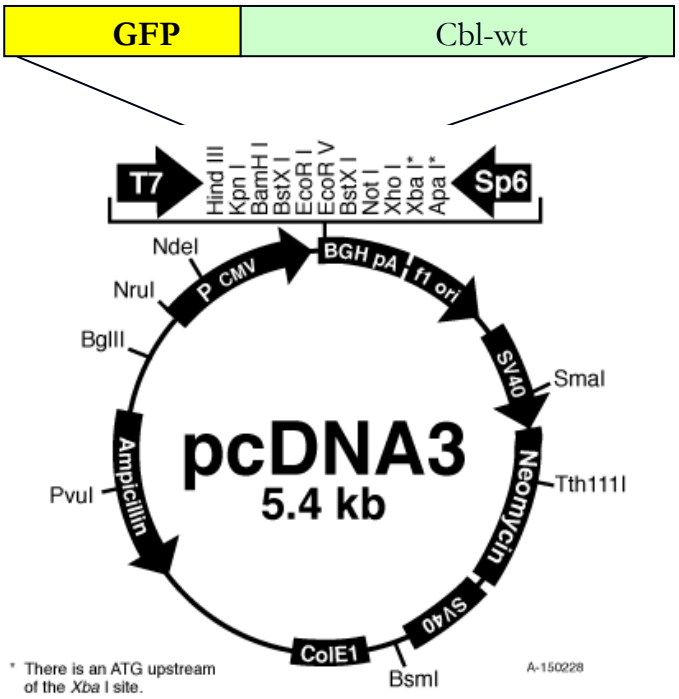

Figure 5. The pcDNA3-GFP Cbl wt template used for mutagenesis procedures. Important features of the construct include the presence of an ampicillin resistance gene, fused cDNA sequences for GFP and Cbl, a cytomegalovirus promoter (P CMV) for strong constitutive expression of the sequences of interest, and a polyA tail for efficient translation termination.

Mammalian cell lines, their passaging and maintenance: This study utilized HEK 293 cells. HEK 293 is an immortalized human embryonic kidney cell line of epithelial morphology (ATCC). The cells express very low levels of EGFR and related receptor tyrosine kinases; by introducing EGFR expression constructs into them, we can evaluate the impact of co-expressed proteins such as Cbl on EGFR homodimers, which are the normal targets for Cbl binding. The HEK 293 cell model system has been proven by the Lill lab to distinguish the activities of wild-type and mutant Cbl proteins in assays of EGFR ubiquitination, downregulation, degradation, and signaling (8, 11, 13).

Cells were passaged regularly at subconfluence, using trypsin/EDTA solution and an established laboratory protocol. The cells were maintained in DMEM containing fetal bovine

serum (FBS) (10%), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin-streptomycin, and 20 mM HEPES, in an atmosphere containing 5% CO_2.

Transient transfection of mammalian cells. Established techniques were used to introduce the mammalian expression constructs into the cells to be tested (8). HEK 293 cells were transfected using a modification of the calcium phosphate precipitation technique (4). DNA precipitates/complexes were normalized for total DNA and promoter input. The GFP epitope tag vector pcDNA3-GFP 2xstop (11) served as a control for its GFP Cbl counterparts.

Before transfection, HEK 293 cells were split at 1:5 ratios (See Mammalian cell lines, their section for more detail) and were 40-50 percent confluent on the day of transfection. Transfection required 1 milliliter of precipitate per 10 cm dish. Components of the 1 ml of precipitate included 450 microliters of water, 4 micrograms of GFP Cbl Wt or mutant GFP Cbl or 3 micrograms of GFP 2xstop, 0.08 microgram of EGFR expression construct, and 50 microliters of 2.5M calcium chloride. Afterwards, 0.5 mL of 2XBBS was added to the tube. Tubes tapped to mix and were set aside for 12-15 minutes for formation of the DNA precipitate. After the precipitation, reaction contents were mixed with a pipette and then 1 milliliter of the precipitate was added to each experimental plate and to the control plates (Cbl wt, and GFP).

Serum-starvation and EGF stimulation of mammalian cells. Prior to assay, the cells were serum-starved for 4-6 hours in DMEM containing 0.5% FBS. This medium facilitates cell survival, but it lacks significant amounts of EGF for receptor signaling during the starvation period. Thus, inactivated receptors will accumulate to high levels at the cell surface and will be

activated as a group upon cell stimulation with EGF (17 nM, which is high but physiologically relevant). Cells expressing EGFR plus the GFP or GFP-Cbl proteins were stimulated (0-40 min) with EGF. This stimulation period spans the time required for surface EGFR to move to lysosomes and undergo degradation. The full range of receptor signaling, from activation to inactivation, can be assessed during this period.

EGFR downregulation assay: Downregulation is the process of receptors leaving the cell surface. The EGFR downregulation assay was developed by Dr. Lill and has been described in detail (14). Concisely: transfected cells were serum-starved to decrease EGFR activation, and the first of four matched transfection plates was harvested for each transfection set without ligand stimulation. Others were stimulated for various time periods with 17nM EGF, which has been shown to result in receptor degradation through ubiquitination. Non-permeabilized, harvested cultures were immunostained using anti-EGFR or isotype-matched control antibodies. All cells then were incubated with a secondary antibody conjugated to a fluorophore emitting light in the low red range. Flow cytometry was used to acquire a quantitative readout of red fluorescence (EGFR signal) from 5000 green cells in each transfection/stimulation tube. Ligand-specific downregulation curves were generated in Microsoft Excel and compared to the positive control curve for wt Cbl-expressing, EGF-stimulated cells.

Results:

My working hypothesis was that the Cbl RF tail mediates the formation of Cbl homodimers through a dimer interface comprising the RF tail of one Cbl molecule and aa 286-354 of a second. To test this hypothesis, I introduced alanine substitution mutations into plasmids carrying full-length Cbl coding sequences fused to green fluorescent protein (GFP) coding sequences. Alanine was chosen as the replacement amino acid because its side chain consists of a methyl group that is conserved among all other amino acids except proline and glycine, and therefore the substitution should not cause significant changes in the protein backbone structure. Instead, only the impact of the loss of the native amino acid side chain should be reflected.

Sequences of the mutagenized constructs were confirmed. Validated constructs were then shown by Western blotting to express the Cbl proteins of expected size (147 kDa: approximately 120kDa for Cbl and 27 kDa for GFP) (Figure 6).

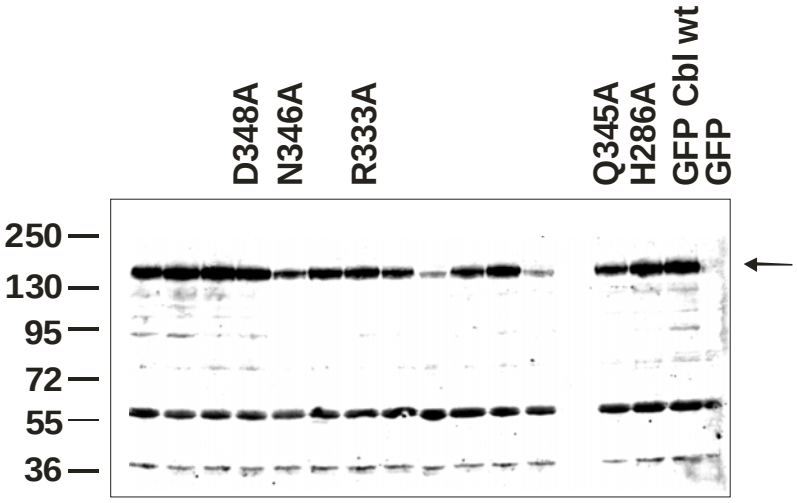

Figure 6. Confirmation that the full-length proteins of interest are expressed in the HEK 293 cell system. Cells were transfected with pAlterMAX-EGFR (0.05 μg/10 cm dish) and the indicated GFP or GFP-Cbl expression constructs (4 μg/10 cm dish). Cultures were processed for lysate preparation. 100 μg of lysate protein was loaded into each lane, resolved by SDS/polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and immunoblotted using an anti-GFP antibody. The positions of molecular mass standard (kDa) are indicated to the left of the panel. The arrow marks the position of the full-length GFP-Cbl fusion proteins.

The candidate alanine substitutions spanned Cbl amino acids 286 through 354. A total of 13 alanine substitution mutants was generated. They included H286A, A310L, D311A, F332A, R333A, F339A, N344A, Q345A, N346A, P347A, D348A, T350A, and E354A. For this project, five constructs were selected for functional analysis. Four of the substitutions, H286A, D348A, R333A and N346A, were predicted to compromise hydrogen bonds within the dimer interface, based on the crystal structure (Figures 2, 4). The fifth mutation, Q345A, should affect a hydrophobic interaction within the crystal interface (Figures 2, 4).

Our examination of the crystal revealed that some of the hydrogen bond interactions at the interface were between amino acid side chains, while others involved peptide backbones. The His286 backbone participates in a hydrogen bonding interaction with the Lys58 side chain; the His 286 side chain also hydrogen bonds with the Asp52 side chain. The substitution of alanine for His286 would maintain the backbone involved in hydrogen bonding to Lys58 while disrupting the interaction between interface residues His286 and Asp52. Any impact of the H286A mutation on EGFR downregulation therefore should be attributed to the disruption of the side chain interaction between His286 and Asp52.

The Asp348 side chain and backbone participate in hydrogen bonding interactions with the Gln 409 side chain and the Lys424 backbone, respectively. We therefore predicted that substituting alanine for Asp348 would impair EGFR downregulation, if the Asp348 sidechain's interaction with Gln409 is critical for Cbl function. If the Asp348 backbone interaction with Lys424's backbone is essential for Cbl activity, our substitution mutant D348A would not reveal this in the downregulation assay.

The Arg333 side chain hydrogen bonds with the side chains of Gln409 and Glu410. If these sidechain interactions are critical for Cbl's downregulation of EGFR, expression of the R333A mutant should compromise receptor downregulation, relative to the amount seen with GFP Cbl wt.

Residue Asn346 interacts via dual hydrogen bonding with Thr426 at the Cbl dimer interface (Figure 4). However, both bonds involve the backbones only of both proteins. We therefore predicted that the N346A mutation will behave like wild-type Cbl in receptor downregulation assays, because the change in side chain should have no affect on the backbone structure.

The final mutant analyzed was Gln345A. In the globular crystal structure (Figure 2), Gln345 appears as an extended finger reaching over the RF tail of the adjacent Cbl molecule. In the crystal (Figure 4), Gln345 participates in a hydrophobic interaction with Glu394 and P428. The latter residue lies within the RF tail but is not evolutionarily conserved (Figure 3). Furthermore, a P428A mutant had been evaluated previously in EGFR downregulation assays and was shown to function like wt Cbl (15). Therefore, the likely impact of the Q345A mutation was unpredictable.

The results of my downregulation assays revealed that mutants H286A and D348A were compromised for EGFR downregulation (Figure 7). Although the GFP and GFP Cbl wt curves were not very widely separated in my graphs, tight experimental error bars allowed me to draw conclusions about significant differences between the control and experimental DNAs (Figure 7). The curves for mutants R333A, Q345A, and N346A were not significantly different from the Cbl wt reference curve. Therefore, only a subset of the tested residues seems important for Cbl-mediated EGFR downregulation from the cell surface.

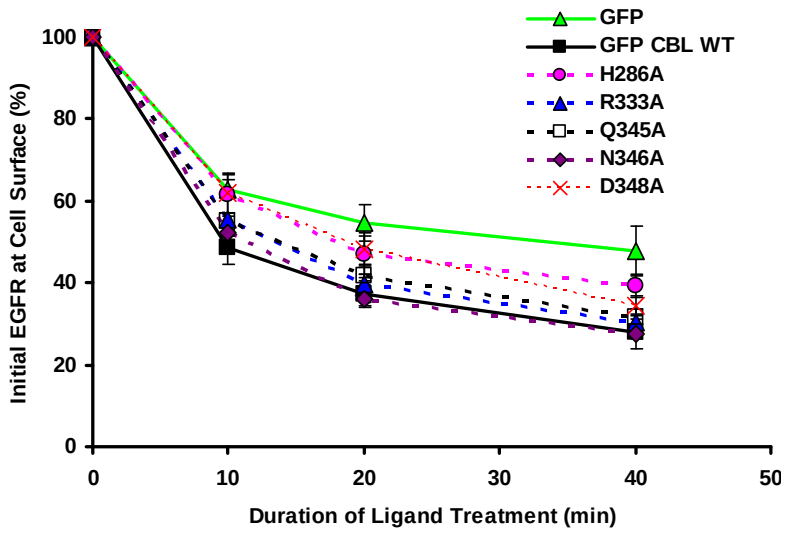

Figure 7. Impact on EGFR downregulation of the GFP-Cbl proteins bearing single amino acid substitutions at the putative Cbl-Cbl dimer interface. HEK 293 cells were transfected with pAlterMAX-EGFR (0.05 μg/10 cm dish) and the indicated GFP or GFP-Cbl expression constructs (4 μg/10 cm dish). At 48 hours post-transfection, the cells were stimulated with EGF for the times shown and then removed from their culture dishes at 4°C, using 0.5 mM EDTA in PBS. The cells were immunostained prior to flow cytometry analysis. In brief, cell samples were processed in triplicate for surface labeling using isotype-matched murine IgG2a anti-EGFR, anti-Syk (negative control) and anti-class I (positive control) antibodies with (*R*)-phycoerythrin-conjugated goat anti-mouse secondary antibody. A Becton Dickinson FACScan with CellQuest Pro software was used to acquire and analyze data from 5000 GFP-positive cells from each sample pool. Readouts reflected the specific and nonspecific binding of each antibody. For each cell population, the specific EGFR MFI (mean fluorescence intensity) was obtained by subtracting the pool's anti-Syk MFI value (nonspecific immunostaining) from its anti-EGFR MFI value.

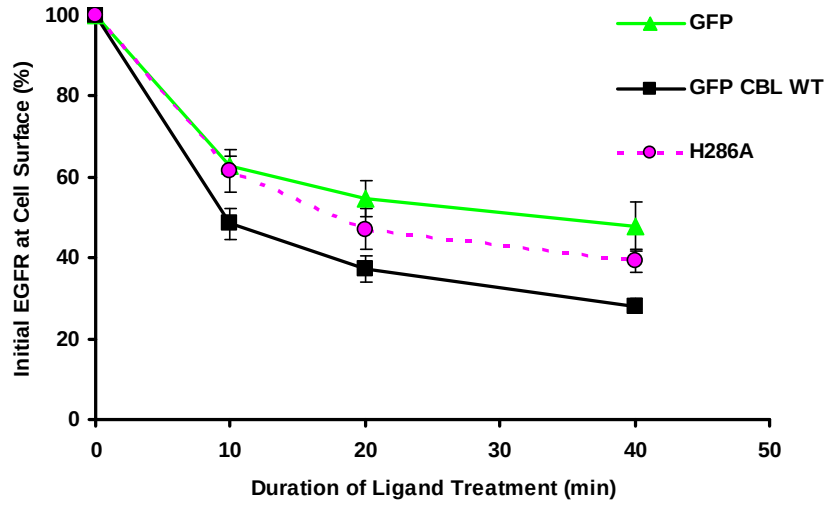

Figure 8. Subset of results from Figure 7, showing the effect of Cbl mutation H286A on EGFR downregulation. The data shown are from three independent experiments. Error bars represent the standard deviation of the means (+/-).

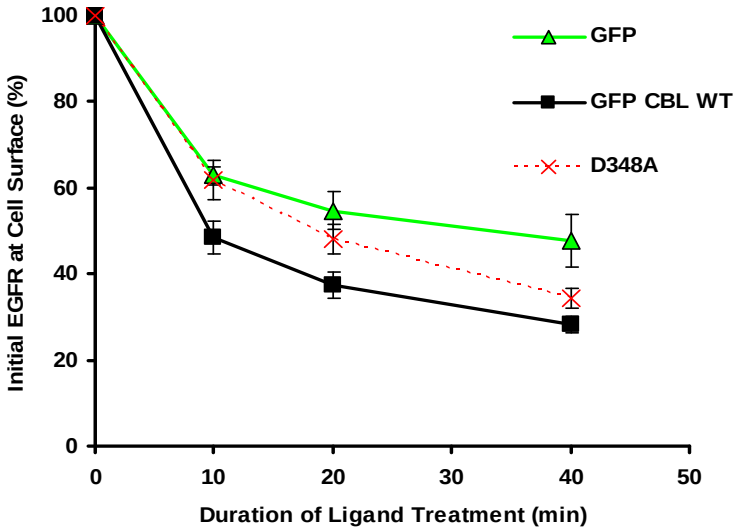

Figure 9. Subset of results from Figure 7, showing the effect of Cbl mutation D348A on EGFR downregulation. The data shown are from three independent experiments. Error bars represent the standard deviation of the means (+/-).

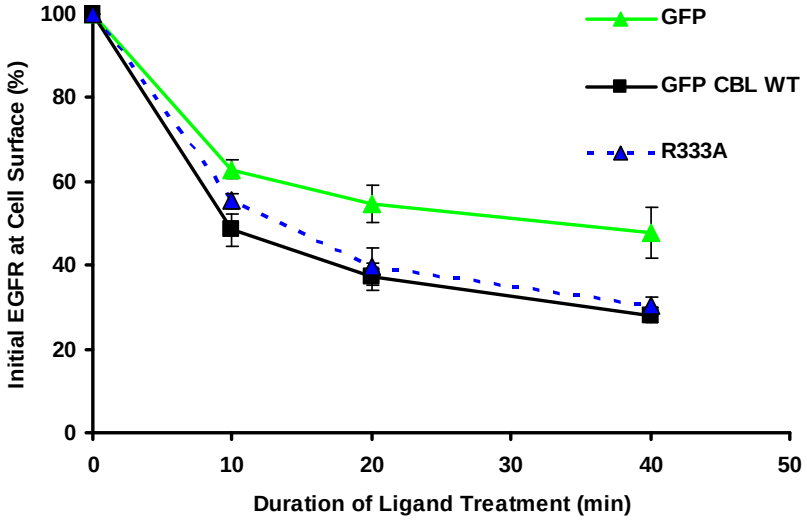

Figure 10. Subset of results from Figure 7, showing the effect of Cbl mutation R333A on EGFR downregulation. The data shown are from three independent experiments. Error bars represent the standard deviation of the means (+/-).

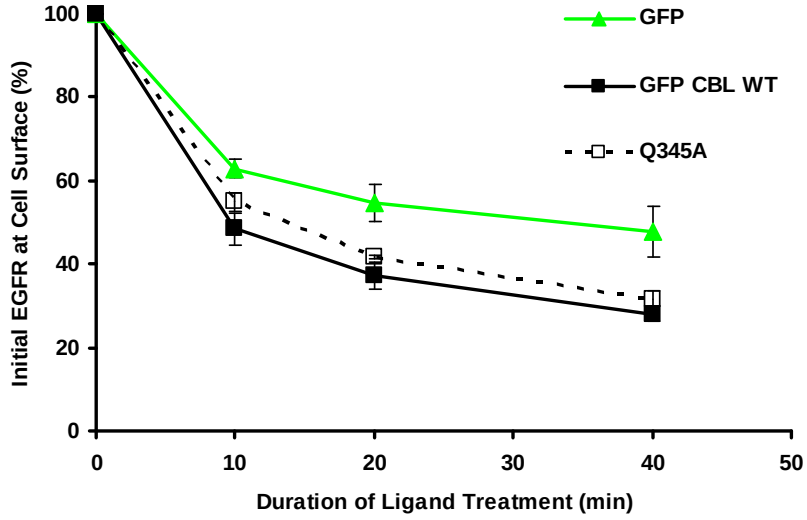

Figure 11. Subset of results from Figure 7, showing the effect of Cbl mutation Q345A on EGFR downregulation. The data shown are from three independent experiments. Error bars represent the standard deviation of the means (+/-).

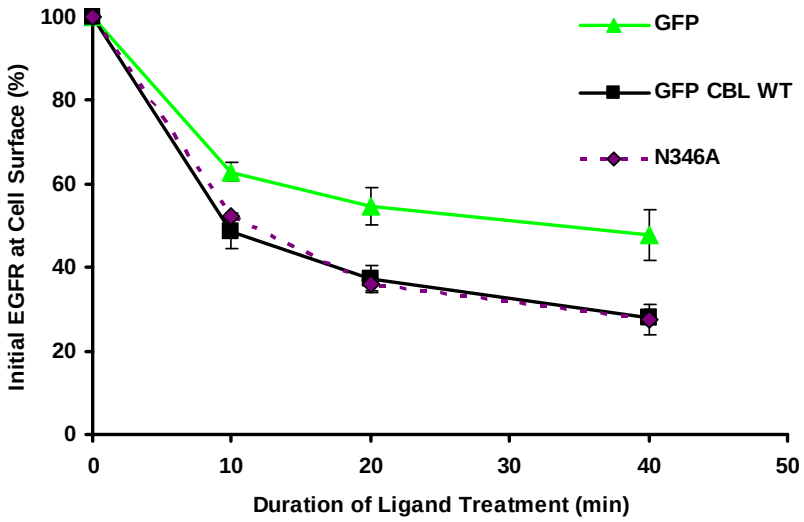

Figure 12. **Subset of results from Figure 7, showing the effect of Cbl mutation N346A on EGFR downregulation.** The data shown are from three independent experiments. Error bars represent the standard deviation of the means (+/-).

Discussion

My data support the hypothesis that a Cbl dimer controls EGFR fate at the level of receptor downregulation from the cell surface. The integrity of putative dimer interface amino acids H286 and D348 is critical for Cbl-mediated EGFR downregulation. In the crystal structure, the side chains of these amino acids engage in intermolecular Cbl-Cbl hydrogen bonds. The integrity of putative dimer interface amino acids R333, Q345, and N346 is not critical for Cbl-mediated EGFR downregulation. However, the residues could affect EGFR trafficking at a stage downstream of receptor downregulation.

Based on the data, I conclude that the H286A and D348A mutants have a defect in internalization and/or recycling, two processes whose combined impact yields a single readout in the downregulation assay. These two mutants may have additional defects further downstream in the lysosomal trafficking pathway of EGFR (Figure 1). However, my assays do not allow us to assign a mutant's defect to any specific stage of endocytosis. Specific internalization, recycling, and degradation assays will be required to address the effect of the H286A and D348A mutants at each stage.

The functional activity of the H286A and D348A mutants suggests which particular interface hydrogen bonds are critical for EGFR downregulation. For H286 (Figure 4), one hydrogen bond involves its peptide backbone and the side chain of Lys58. The second hydrogen bond is between the H286 side chain and the Asp52 sidechain. Only the second hydrogen bond should be affected by replacing H286 with alanine; therefore, this must be the important interaction for Cbl-mediated EGFR downregulation. For D348, one hydrogen bond involves the peptide backbone and the backbone of Lys424. The second bond involves the side chains of D348A and

Gln409, and only this bond should be disrupted by substitution of alanine for D348. Therefore, this must be the critical hydrogen bond of D348 that is necessary for normal EGFR downregulation.

The R333A, Q345A and N346A mutants had no detectable impact on EGFR downregulation, so they are fully functional for the removal of EGFR from the cell surface. I conclude that the hydrogen bonds between paired residues Arg333::Gln409, Arg::Glu410, and Gln346::Thr426 are dispensable at the Cbl dimer interface for wild type levels of EGFR downregulation. The results for the Gln346::Thr426 were as predicted, as the side chain substitution was expected to have no impact on the hydrogen bond involving only the peptide backbones of the residues.

The wild type activity of the R333A, Q345A and N346A mutants in the downregulation assay does not mean that the mutants promote wild type levels of EGFR degradation. It is possible that they affect EGFR trafficking at a stage downstream of receptor downregulation. In this case, the internalized receptors could remain inside the cell as with Cbl wt, but their movement toward lysosomes might be arrested at the level of early endosome formation or fusion (15), MVB formation, or compartment acidification. Fixed cell and live cell microscopy for the detection of EGFR/Cbl complexes will help to address this question.

In conclusion, my results support the hypothesis that amino acids within the Cbl dimer interface are critical for Cbl-regulated EGFR downregulation. Future investigations will map the stage(s) of endocytosis at which they act.

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